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(54) Title: INDUCIBLE NITRIC OXIDE SYNTHASE AND GENE THEREFOR (57) Abstract The invention provides synthetic DNAs which are capable of expressing peptide proteins toxic to insects when incorporated within the genome of a suitable host organism, e.g. a baculovirus, the proteins being the same as or structurally and functionally similar to peptide toxins derived from molluscs, e.g. <i>Conus</i> spp.		

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INDUCIBLE NITRIC OXIDE SYNTHASE AND GENE THEREFOR.

The invention relates to a novel nitric oxide (NO) synthase, DNA coding therefor, methods for detection of the NO synthase and the DNA, a method for screening for compounds capable of inhibiting or stimulating the NO synthase, and the compounds thereby identified.

The demonstration in 1987 of the formation of NO by an enzyme in vascular endothelial cells opened up what can now be considered as a new area of research (for review, see Moncada *et al.* (1991) Pharmacological Reviews, vol. 43, no. 2, 109-142). NO is synthesised from the amino acid L-arginine by the enzyme NO synthase.

The synthesis of NO from L-arginine by NO synthase is now recognized as an important pathway for regulating the function of a wide variety of cells and tissues. For example, in the blood vessel wall, NO is synthesised by the vascular endothelium to regulate smooth muscle tone and thus blood pressure. Nitric oxide synthase is also present in the central nervous system, where NO is a neurotransmitter/neuromodulator mediating the action of glutamate on NMDA receptors and mediating/modulating transmission in nerves previously recognised as nonadrenergic and noncholinergic. NO can also act as an autocrine regulator on some cells, including platelets, where it modulates their activation. NO generated by activated macrophages is also an important effector molecule in host defence. In this role, NO has been shown to possess anti-tumour and anti-microbial activity against various parasites *in vitro* and *in vivo*.

NO synthases can be classified into two types, namely inducible and constitutive NO synthases. NO synthase activity is constitutively expressed in a variety of cells including endothelial cells, neurons, platelets, adrenal gland cells, and endocardium cells. In contrast, NO synthase is inducible in macrophages, hepatocytes, Kupffer cells, vascular smooth muscle and vascular endothelium following activation with endotoxin and/or cytokines.

More recently, NO synthase has been shown to be induced in rabbit articular chondrocytes (Stadler *et al.* (1991) J. Immunol. 147, 3915-3920 and Palmer *et al.* (1992) Biochem. Biophys. Res. Commun. 188, 209-215). We have observed that in human chondrocytes the enzyme is induced by interleukin-1 β (IL-1 β). The induction of NO synthase in human cells has previously only been reliably shown in hepatocytes (Nussler *et al.* (1992), J. Exp. Med. 176, 261-264). The induction of NO synthase in chondrocytes is likely to have a role in joint disease (i.e. arthritic disease). IL-1 β concentrations are increased in the inflamed joint and under these conditions NO synthase is likely to be induced.

The NO synthase enzymes comprise a family that can be distinguished on the basis of comparative DNA sequence analysis. Sequences have been reported for the constitutive neuronal NO synthase cDNAs from rat and man (Bredt *et al.* (1991) Nature 351, 714-718 and Nakane *et al.* (1993) Febs. Lett. 316, 175-180), the constitutive endothelial NO synthase cDNAs from bovine and human tissue (Lamas *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 6348-6352; Janssens *et al.* (1992) J. Biol. Chem. 267, 14519-14522; Sessa *et al.* (1992) J. Biol. Chem. 267, 15274-15276; Marsden *et al.* (1992) Febs. Lett. 307, 287-293) and an inducible NO synthase cDNA from a rodent macrophage line (Lyons *et al.* (1992) J. Biol. Chem. 267, 6370-6374; Xie *et al.* (1992) Science 256, 225-228; and Lowenstein *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 6711-6715). A comparison of the deduced protein sequences derived from the three classes of NO synthase enzymes shows, overall, approximately 50-60% similarity. For all three enzymes, the highest degree of similarity occurs around a conserved region within the first third of the molecule, thought to represent the L-arginine binding site.

Although two distinct forms of the constitutive enzyme have been described at the molecular level, with high identity between the rat, and human neuronal forms and between the bovine and human endothelial forms, only the murine

macrophage form of the inducible NO synthase has been similarly characterized.

In the present invention, a novel human inducible NO synthase has been characterized. The full length cDNA encoding the NO synthase has been cloned and expressed using human articular chondrocytes activated with IL-1 β . The NO synthase enzyme has utility in a number of settings as described hereinafter, but is of particular value in a research environment in which it can be used in a screen or assay to identify compounds that are capable of inhibiting or stimulating the activity of the enzyme. Such compounds have utility in the clinic for the treatment of indications as described hereinafter.

The present invention provides an NO synthase having the sequence of SEQ ID NO: 2, or an NO synthase having a sequence at least 85% identical to the sequence of SEQ ID NO: 2. The NO synthase may, for example, have a sequence at least 90%, at least 95%, at least 98% or at least 99% identical to SEQ ID NO: 2. The NO synthase is generally of human origin, preferably human chondrocyte origin.

The expression 'NO synthase' includes the full length cDNA encoding the NO synthase and any fragment thereof. The fragment of the NO synthase may be a fragment at least 6 amino acids in length. The length of the fragment may be, for example, at least 8, at least 12, at least 24, at least 48 or at least 96 amino acids, and is intended to include the fragment corresponding to the active domain of the enzyme, i.e. the domain which catalyses the synthesis of NO from L-arginine.

The NO synthase is generally in substantially pure form. Preferably, the NO synthase in substantially pure form comprises a preparation in which at least 90%, at least 95%, at least 98% or at least 99% of the weight of protein in the preparation is the NO synthase of the invention.

The NO synthase will usually be obtained by recombinant DNA techniques. However, the NO synthase may be obtained using biochemical purification of the protein from its

natural origin.

The invention provides a DNA molecule encoding the NO synthase. The DNA molecule may contain the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1. Alternatively, the DNA molecule may contain a sequence at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1.

A person of ordinary skill in the art would know how to obtain a DNA molecule according to the invention using the sequences disclosed herein, without undue experimentation. The DNA molecules according to the invention could be produced by various means, such as, for example, DNA synthesis, or more preferably, by recombinant DNA techniques. Techniques for synthesising DNA molecules are described by, for example, Wu *et al* (Prog. Nucl. Acid. Res. Molec. Biol. 21, 101-141 (1978)). Techniques for constructing recombinant molecules are described by Sambrook *et al* (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

The present NO synthase may be obtained by making a library of replicable expression vectors. The library may be created by cloning genomic DNA or, more preferably, cDNA into a parent vector. The cDNA may be obtained using the poly A⁺ mRNA of a cell (e.g. a chondrocyte) in which NO synthase production has been induced, e.g. by a cytokine such as IL-1 β . The library is screened for members containing the desired nucleic acid sequence, e.g. by means of a DNA probe or antibody. A probe having a sequence identical to a portion of the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1 or exactly complementary to a portion of the sequence nucleotides 226 to 3687 of SEQ ID NO: 1 may be used to identify an NO synthase coding sequence not identical to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1 (e.g. from 85% to 99% identical to nucleotides 226 to 3687 of SEQ ID NO: 1) by low stringency hybridization.

A replicable expression vector is a vector which

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contains the appropriate origin of replication sequence for replication of the vector and the appropriate sequences for expression of the foreign nucleotide sequence in the vector. The sequences for expression of a foreign sequence will generally include a transcription promoter operably linked to the foreign sequence. The term "operably linked" refers to a linkage in which the promoter and foreign sequence are connected in such a way to permit expression of the foreign sequence. The transcription promoter sequence may be part of the parent vector sequence into which the foreign sequence is inserted. Alternatively, the promoter sequence may be a native promoter sequence of a gene encoding an NO synthase of the invention, so that the NO synthase is inducible by IL-1 β . A vector may be, for example, a plasmid, virus or phage vector. A vector may contain one or more selectable markers, for example an ampicillin resistance gene in the case of a bacterial vector or a neomycin resistance gene in the case of a mammalian vector. A foreign gene sequence inserted into a vector may be transcribed in vitro or the vector may be used to transform or transfect a host cell.

According to one embodiment of the invention, there is provided a host cell transformed or transfected with a vector. A vector and host cell will be chosen so as to be compatible with each other, and may be prokaryotic or eukaryotic. A prokaryotic host may, for example, be E. coli, in which case the vector may, for example, be a bacterial plasmid or a phage vector. A eukaryotic host may, for example, be a yeast (e.g. S. cerevisiae) a chinese hamster ovary (CHO) cell or an insect cell (e.g. Spodoptera frugiperda). When the host is an insect cell, the vector is generally a baculovirus vector (reviewed by Luckow and Summers (1988) in BIO/TECHNOLOGY, Vol. 6, 47-55).

An NO synthase according to the invention may be produced by a method comprising

- (a) culturing a host cell under conditions in which the cell expresses the NO synthase; and

(b) recovering the NO synthase from the culture.

In this method, expression of NO synthase may be induced by a cytokine such as IL-1 β . The NO synthase may be recovered from either the host cell or, where the NO synthase is secreted by the host cell, from the culture supernatant.

The invention includes a an oligonucleotide fragment having a sequence of a portion of a DNA molecule encoding an NO synthase of the invention, and an oligonucleotide fragment having a sequence at least 85%, at least 90%, at least 95%, at least 98%, or at least 99%, identical to a sequence encoding an NO synthase of the invention or fragment thereof. The fragment is at least 12 nucleotides in length, e.g. at least 15, at least 18, at least 30, at least 60, at least 180, or at least 720 nucleotides in length. The fragment may be single or double stranded. When the fragment is single stranded, it may have a sequence from either a sense or antisense strand of a DNA molecule encoding an NO synthase of the invention. An antisense fragment may be useful in the therapeutic treatment of a disease involving over-expression of NO synthase, whilst a full-length expression fragment may be of use in treatment of conditions requiring stimulation of the NO synthase, for example treatment of some viral diseases or solid tumours.

The fragment will generally be DNA, although other types of nucleic acid may be used, for example RNA or modified DNA. A number of different types of nucleic acid modification are known in the art. These include methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule.

The oligonucleotide fragment may be an oligonucleotide probe or an oligonucleotide polymerase chain reaction (PCR) primer, which will hybridise to a nucleic acid molecule (e.g. a DNA or RNA molecule) encoding an NO synthase of the invention. The probe or a pair of primers may be used to detect or quantitatively determine the nucleic acid sequence. This has diagnostic utility in detecting and quantitatively determining NO synthase mRNA associated with a

disease state, for example arthritic disease, high blood pressure, disorders of the central nervous system, and cancers such as breast cancer.

A fragment which is a probe or primer may carry a revealing label, such as ^{32}P , digoxigenin or biotin. Preferably, the probe or primer will specifically hybridise only to its target sequence, e.g. a portion of SEQ ID NO: 1, and not to other sequences. However, it will be appreciated that this will not always be the case, and the probe or primer may only be selective for its target sequence. A probe or primer which hybridises only to its target sequence will generally be exactly complementary to the target sequence (e.g. a portion of the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1) whereas a probe or primer which is only selective may have a sequence which is, for example, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% complementary to the target sequence. A probe which is not exactly complementary to its target sequence has utility in the identification of new NO synthase nucleotide sequences having a sequence similar to the sequence of nucleotides 226 to 3687 of SEQ ID NO: 1. A fragment which is a probe or primer may have from 12 to 60 nucleotides, e.g. from 12 to 40 nucleotides, or from 15 to 30 nucleotides.

Primers for PCR are generally provided as a pair. A first primer hybridises to a sense sequence 3' to the sequence to be amplified and a second primer hybridises to an antisense sequence 5' to the sequence to be amplified. This allows synthesis of double stranded DNA representing the region between the two primers.

Thus, the invention includes a method of amplifying a target nucleic acid sequence present in a nucleic acid encoding an NO synthase of the invention, which method comprises carrying out PCR employing a primer of the invention. Such a method generally comprises carrying out cycles of

- (a) denaturing double stranded DNA containing the target sequence to obtain single stranded DNA;

(b) hybridizing a first primer to a sense strand 3' to the target sequence, and hybridising a second primer to an antisense strand 5' to the target sequence; and

(c) synthesising DNA from the first and second primers.

The number of cycles is suitably from 10 to 50, preferably 20 to 40, more preferably 25 to 35. The method may be carried out starting from a double stranded nucleic acid (e.g. dsDNA) or a single stranded nucleic acid (e.g. mRNA). The target sequence may be a complete NO synthase encoding sequence or a partial NO synthase encoding sequence.

As will be appreciated by a person skilled in the art, the method described above is based upon the well-known polymerase chain reaction (PCR) method. A skilled person would know of detailed protocols for carrying out PCR and reverse transcriptase-PCR (RT-PCR). Reviews of PCR are provided by Mullis (1986) Cold Spring Harbor Symp. Quant. Biol. 51, 263-273; Saiki *et al.* (1985) Bio/Technology 3, 1008-1012; and Mullis *et al.* (1987) Meth. Enzymol 155, 335-350.

An oligonucleotide probe according to the invention has utility in detecting or quantitatively determining a nucleic acid (e.g. a DNA or RNA) encoding an NO synthase according to the invention. Conventional methods for detecting or quantitatively determining a nucleic acid may be used, for example in situ hybridization, Southern blotting or Northern blotting. Accordingly, there is provided a method of detecting or quantitatively determining in a sample a target nucleic acid sequence present in a nucleic acid encoding an NO synthase according to the invention, which method comprises

- (a) contacting the probe with the sample; and
- (b) detecting or quantitatively determining hybridization of the probe with any target nucleic acid sequence present in the sample.

The sample containing the target nucleotide sequence may, for example, be a tissue specimen, a tissue extract or

cell extract from a patient suffering from a disease associated with abnormal NO synthase activity such as arthritis, high blood pressure, a disorder of the central nervous system, or a cancer such as breast cancer. Alternatively, the sample may, for example, be a sample produced as a result of a recombinant DNA procedure, in which case the sample may be a vector or an extract of host cells. The target nucleic acid sequence may be a complete NO synthase encoding sequence or a partial NO synthase encoding sequence.

A preferred method of detecting or quantitatively determining a target nucleic acid sequence in a sample comprises

- (i) subjecting the sample to gel electrophoresis to separate the nucleic acids;
- (ii) transferring the separated nucleic acids onto a solid support (e.g. a nitrocellulose support) by blotting; and
- (iii) hybridising a probe according to the invention to the target nucleic acid sequence.

A probe can be used in an in situ hybridization procedure to locate a nucleic acid sequence encoding an NO synthase of the invention. This can be done to determine the spatial distribution of NO synthase encoding DNA or mRNA sequences in a cell or tissue. In the case of mRNA detection, the tissue is gently fixed so that its RNA is retained in an exposed form and the tissue is then incubated with a labelled complementary probe.

A polypeptide fragment of the present invention has utility in, for example, producing antibodies against an NO synthase.

Thus, the invention includes an antibody specific for an NO synthase according to the invention. The antibody has utility in detecting and quantitatively determining NO synthases, and hence is useful in diagnosis of diseases associated with NO synthase, such as the diseases listed herein. An antibody of the present invention may also be of use

in identifying which NO synthase enzyme is responsible for a particular condition. The antibody also has utility in production of NO synthases by recombinant DNA procedures, for example in detection of positive clones containing a target sequence. Furthermore, the antibody may be of use as a therapeutic agent.

The antibody is preferably monoclonal, but may also be polyclonal. The antibody may be labelled. Examples of suitable antibody labels include radiolabels, biotin (which may be detected by avidin or streptavidin conjugated to peroxidase), alkaline phosphatase and fluorescent labels (e.g. fluorescein and rhodamine). The term "antibody" is used herein to include both complete antibody molecules and fragments thereof. Preferred fragments contain at least one antigen binding site, such as Fab and F(ab')₂ fragments. Humanised and chimaeric antibodies and fragments thereof are also included within the term "antibody".

The antibody is produced by raising antibody in a host animal against an NO synthase according to the invention or an antigenic epitope thereof (hereinafter "the immunogen"). Methods of producing monoclonal and polyclonal antibodies are well-known. A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified. A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein, Nature 256, 495-497, 1975).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an

allogenic host or immunocompromised host. Human antibody may be prepared by in vitro immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

The invention includes a method of detecting or quantitatively determining in a sample an NO synthase of the invention, which method comprises

- (a) contacting the sample with an antibody of the invention; and
- (b) detecting or quantitatively determining the binding of the antibody.

A preferred method for detecting or quantitatively determining an NO synthase is Western blotting. Such a method can comprise the steps of

- (i) subjecting a sample containing a target NO synthase to gel electrophoresis to separate the proteins in the sample;
- (ii) transferring the separated proteins onto a solid support (e.g. a nitrocellulose support) by blotting; and
- (iii) allowing an antibody according to the invention which has been labelled to bind to the target NO synthase.

Preferred methods of quantitative determination are ELISA (enzyme-linked immunoassay) methods such as a non-competitive ELISA methods. Typically, an ELISA method comprises the steps of

- (i) immobilising on a solid support an unlabelled

- antibody according to the invention;
- (ii) adding a sample containing the target NO synthase such that the NO synthase is captured by the unlabelled antibody;
 - (iii) adding an antibody according to the invention which has been labelled; and
 - (iv) quantitatively determining the amount of bound labelled antibody.

An antibody of the invention may be employed histologically for in situ detection of an NO synthase, e.g. by immunofluorescence or immunoelectron microscopy. In situ detection may be accomplished by removing a histological specimen from a patient, and allowing a labelled antibody to bind to the specimen. Through use of such a procedure, it is possible to determine not only the presence of an NO synthase but also its distribution.

An antibody of the invention may be used to purify a target NO synthase. Conventional methods of purifying an antigen using an antibody may be used. Such methods include immunoprecipitation and immunoaffinity column methods. In an immunoaffinity column method, an antibody in accordance with the invention is coupled to the inert matrix of the column and a sample containing the target NO synthase is passed down the column, such that the target NO synthase is retained. The NO synthase is then eluted.

The sample containing the target NO synthase used in the detection, determination and purification methods may be a tissue specimen, a tissue extract or a cell extract from a patient suffering from a disease associated with NO synthase, such as a disease listed above. Alternatively, the sample may be one produced as a result of recombinant DNA procedures, e.g. a vector or an extract of host cells.

An NO synthase of the invention is useful for screening for substrates which inhibit or stimulate the enzyme. The invention includes a method for identifying a substrate which inhibits or stimulates the NO synthase, which method

comprises

- (a) incubating the NO synthase with the substrate;
- (b) measuring the activity of the NO synthase; and
- (c) comparing the activity measured in (b) above with the activity of the NO synthase in the absence of the substrate.

For example, the activity of a substrate as an inhibitor or stimulator of the NO synthase of the present invention can be determined by an assay in which activated chondrocytes are incubated with the substrate, and NO synthase activity recorded using a dual wavelength spectrophotometer, reading at 401 and 421.

The invention also extends to substrates identified by the use of a screen hereinbefore described. Preferably the substrate is a chemical molecule of relatively low molecular weight, for example, less than about 1000. Examples of suitable classes of molecule include arginine analogues and isothioureia derivatives. Alternatively, the substrate can be a macromolecule, for example an antibody.

The invention also includes an enzyme-substrate complex which comprises an NO synthase as described herein and a substrate capable of inhibiting or stimulating the activity of the NO synthase. The enzyme-substrate complex optionally exists in an ex vivo situation.

A substrate which inhibits or stimulates the NO synthase enzyme is of utility in medical therapy. Inhibition of the inducible NO synthase may have many clinical utilities, for example in the treatment of septic shock and in particular in the treatment of hypotension associated therewith, in therapy with cytokines such as TNF, IL-1 and IL-2 or therapy with cytokine-inducing agents, for example 5, 6-dimethylxanthenone acetic acid, as an adjuvant to short term immunosuppression in transplant therapy, in patients suffering from inflammatory conditions in which an excess of NO contributes to the pathophysiology of the condition, for example adult respiratory distress syndrome and myocarditis, and in autoimmune and/or

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inflammatory conditions such as arthritis and rheumatoid arthritis. Inhibition of the NO synthase enzyme may also be of use in the treatment of cerebral ischemia, CNS trauma, epilepsy, AIDS dementia, chronic neurodegenerative disease and chronic pain, and conditions in which non-adrenergic non-cholinergic nerve may be implicated such as priapism, obesity and hyperphagia. On the other hand, stimulation of the inducible NO synthase would lead to increased NO levels in the body and may be of use in treating parasitic and/or viral diseases, and killing tumour cells.

There is also provided within the scope of the present invention a pharmaceutical formulation which comprises one or more of a substrate identified using the present invention, an NO synthase as described herein, or an antibody of the present invention in combination with a pharmaceutically acceptable carrier or diluent therefor, and optionally one or more further therapeutic agents.

Formulations comprising a substrate, include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular), rectal and topical (including dermal, buccal, sublingual and intraocular) administration although the most suitable route may depend upon for example the condition and disorder of the recipient. Formulations comprising an NO synthase as described herein, or an antibody are those suitable for parenteral administration. All formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association a substrate, an NO synthase as described herein, or an antibody ("active ingredient") with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

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Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration may be presented as a suppository with the usual carriers such as cocoa butter or polyethylene glycol.

Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges comprising the active ingredient in a flavoured basis such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a basis such as gelatin and glycerin or sucrose and acacia.

Preferred unit dosage formulations are those containing an effective dose, as hereinbelow recited, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of

this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The substrates are preferably administered orally or via injection at a dose of from 0.1 to 500mg/kg per day. The dose range for adult humans is generally from 5mg to 35g/day and preferably 5mg to 2g/day. Tablets or other forms of presentation provided in discrete units may conveniently contain an amount of compound of the invention which is effective at such dosage or as a multiple of the same, for instance, units containing 5mg to 500mg, usually around 10mg to 200mg.

The NO synthase as described herein or antibody are administered parenterally at a dose of from 1 to about 100 mg for an adult patient, preferably 1 - 10 mg, usually administered daily for a period between 1 and 30 days. A two part dosing regime may be preferable wherein 1 - 5 mg are administered for 5 - 10 days followed by 6 - 15mg for a further 5 - 10 days.

The precise amount of active ingredient administered to a patient will be the responsibility of the attendant physician. However the dose employed will depend on a number of factors, including the age and sex of the patient, the precise disorder being treated, and its severity. Also the route of administration may vary depending on the condition and its severity.

The present invention will now be described by way of example only, and is not intended to be limiting thereof.

EXAMPLE 1

Description of the Drawings

Figure 1 shows the results of Northern blot analysis of inducible NO synthase specific mRNA from human chondrocytes. PolyA⁺ mRNA (0.25 µg), extracted from induced and uninduced cells, was electrophoresed through a formaldehyde-agarose gel

and transferred to a nylon membrane. The blot was hybridized with a full-length cDNA probe labelled with digoxigenin and washed under high stringency conditions. A positively hybridizing band at 4.4 kb) is apparent only in track 2 (induced). Track 1 was loaded with the same amount of polyA⁺ mRNA from uninduced cells and shows no positively hybridizing band. The positions of molecular mass markers are indicated.

Figure 2 shows the results of expression of recombinant human chondrocyte inducible NO synthase in CHO cells. CHO cells were transfected with pSVL-NO containing the full-length cDNA for human iNOS, and NO synthase activity assayed as NO in the culture supernatant after (A) 24 h or (B) 96 h. Controls were parent cells alone (i.e. untransfected; closed box) and an unrelated CHO-recombinant (pSVLS; hatched box). Only the pSVL-NO recombinant CHO cells produced significant NO in the medium (open box) and this was blocked by incubation with the NO synthase inhibitors L-N-iminoethyl-ornithine L-NIO) (100 μ M) and N-guanidino-monomethyl-L-arginine (L-NMMA) (100 μ M).

MATERIALS AND METHODS

Cell culture and isolation of mRNA

Human chondrocytes were isolated and cultured. In order to induce NO synthase activity, cells were incubated with IL-1 β (1 ng/ml) for 24 h. The methods of culture and induction used were analogous to those described in relation to rabbit chondrocytes by Stadler *et al.* (1991) J. Immunol 147, 3915-3920 and Palmer *et al.* (1992) Biochem. Biophys. Res. Commun. 188, 209-215. Cells were harvested with trypsin, washed with growth medium, pelleted and frozen at -70°C. PolyA⁺ mRNA was extracted with a Micro Fast-Track kit (Trade Name, Invitrogen) from chondrocytes (1-2x10⁶ cells) incubated for 24 h with or without IL-1 β (1 ng/ml). Typically 1-2 μ g polyA⁺ mRNA was purified from 1x10⁶ cells.

The murine macrophage cell line J774 was cultured and induced to express NO synthase with interferon γ and

lipopolysaccharide from Escherichia coli strain 026.B6 as described previously (Cunha et al (1993) J. Immunol 150, 1-6. PolyA⁺ mRNA was extracted as described above.

Dihydrofolate reductase⁻ (DHFR⁻) CHO cells were maintained in 75 cm flasks in Dulbecco's MEM (Trade Name, Gibco), 10% foetal calf serum (FCS), 1 mM L-glutamine, non essential amino acids, antibiotics, 100 μ M hypoxanthine and 16 μ M thymidine. pSVL transfected cells were cultured in the absence of hypoxanthine and thymidine, but in the presence of dialysed FCS and 100 μ M methotrexate. For experimental purposes, cells were trypsinised from the flasks, washed once in phosphate buffered saline (PBS) and plated at 10⁶ cells/well in 12 well plates in 3 ml of appropriate culture medium. L-N-iminoethyl-ornithine (L-NIO) or N-guanidino-monomethyl-L-arginine (L-NMMA) was added to some cultures to a final concentration of 100 μ M. All cultures were then incubated at 37°C in a humidified 5% CO₂ atmosphere. Samples (100 μ l) were removed from triplicate cultures at 24 h intervals and stored at 4°C before determination of NO by chemiluminescence (Palmer et al. (1987), Nature 327, 524-526)

Identification of inos by reverse transcriptase-polymerase chain reaction (RT-PCR) and construction of a cDNA library

The primers BB3: 5'-CGGGATCCGGNACNGGNATHGCNCCNTT-3' (SEQ ID NO: 3) and BB4: 5-GCGAATTCNCCRCANACRTADATRTG-3' (SEQ ID NO: 4) were used to amplify random primed cDNA generated from human induced and uninduced chondrocyte polyA⁺ mRNA by the polymerase chain reaction (PCR) using a Gene Amp RT-PCR kit (Trade Name, Perkin-Elmer Cetus) following the manufacturers recommended procedures. The following conditions were used: denaturation 96°C, 35s; anneal 55°C, 2 min; and extension 72°C, 3 min for 30 cycles. For a reaction volume of 100 μ l, 50 ng of polyA⁺ mRNA was used with 50 ng of each primer. PCR products were digested with EcoRI-BamHI, resolved by agarose gel electrophoresis, purified and ligated into EcoRI-BamHI digested Bluescript pBS SKII⁺ (Stratagene) by standard methods (Sambrook

et al. (1989) Molecular cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

RT-PCR was carried out on polyA⁺ mRNA extracted from induced J774 cells using oligonucleotide primers derived from, the RAW 264.7 sequence (Lyons *et al.* (1992) J. Biol. Chem. 267 6370-6374; Xie *et al.* (1992) Science 256, 225-228; and Lowenstein *et al.* (1992) Proc. Natl. Acad. Sci. USA 89, 6711-6715). The primers, AL14: 5'-ACGGAGAAGCTTAGATCTGGAGCAGAAGTG-3' (SEQ ID NO: 5) and AL15: 5'-CTGCAGGTTGGACCACTGGATCCTGCCGAT-3' (SEQ ID NO: 6) generated a 630 bp band corresponding to the 5-end of the gene. PCR products were digested with *Hind*III and *Bam*HI purified and cloned into *Hind*III-*Bam*HI digested Bluescript pBS SKII+ (Sambrook *et al.*, supra).

PolyA⁺ mRNA (1.5 µg) isolated from chondrocytes activated with IL-1β (1 ng/ml for 24 h) was used to generate a cDNA library in the bacteriophage lambda ZapII (Stratagene). Both random and oligo-dT primers were used in the cDNA synthesis and 5x10⁵ independent recombinant phage were generated. Phage were amplified once, and 10⁶ plaques plated out and screened (in duplicate) using standard techniques (Sambrook *et al.*, supra) with the 630 bp fragment from the murine inducible *inos* gene labelled with [α³²P].

Hybridization and DNA sequencing

Blot and plaque hybridizations were carried out on GeneScreen Plus hybridization membranes (Trade Name, DuPont). Northern blot analysis was carried out using digoxigenin labelled probes (Boehringer Mannheim) after electrophoresis and transfer of mRNA from denaturing formaldehyde-agarose gels (Sambrook *et al.*, supra).

Recombinant DNA was sequenced using double standard DNA as template (Stephen *et al.* (1991) Nucleic Acids Res. 24, 7463-7464). An overlapping series of deletions was made in template DNA (Henikoff (1984) Gene 28, 351-359 using the exonuclease III kit (Pharmacia). Sequencing was carried out using universal primer, [α³⁵S] dATP and wedge gels (Sanger *et al.* (1983) Proc. Natl. Acad. Sci. USA 74, 5463-5467). Clones

were sequenced with modified T7 DNA polymerase (Tabor and Richardson (1987) Proc. Natl. Acad. Sci. USA 84, 4767-4771). To resolve compression artifacts (Mizusawa *et al.* (1986) Nucleic Acids Res. 14, 1319-1324) some clones were sequenced with 7-deaza-2-dGTP (Pharmacia). Gaps in the sequence were filled in using synthetic oligonucleotides made on a Milligen 7500 DNA synthesizer (Trade Name, Millipore) as specific primers (Charles *et al.* (1986) Nucleic Acids. Res, 14, 2201-2213).

Expression of a stable CHO cell line expressing NOS

DHFR⁻ CHO cells were co-transfected with 10 µg of the full-length cDNA for the human inducible NO synthase, pSVL-NO, cloned as an XbaI fragment into the vector pSVL (Pharmacia, UK) and with 1 µg of the DHFR encoding plasmid pRDN2 (Dr. N. Sharp, Wellcome Research Laboratories, Beckenham, Kent). Cells were seeded at 10⁶ per 100 cm petri dish and individual recombinants cloned by dilution cloning. Individual clones were assayed for the ability to increase the NO₂- concentration in growth medium. One cell line, CHO-INOS-20, expressing the highest levels of inducible NO synthase was selected for further study.

RESULTS

Cloning of an inducible human nos gene

The strategy used to clone the human chondrocyte inducible NO synthase cDNA was based on the finding that significant levels of NO synthase activity can be induced in these cells by IL-1β. Northern blotting showed the presence of a 4.4 kb NOS-specific band in mRNA extracted from induced cells that was absent in uninduced cells (Fig. 1). By using RT-PCR and degenerate oligonucleotide primers, a 350 bp fragment of the rabbit chondrocyte iNOS cDNA was cloned and sequenced which had greater than 90% identity with the murine inducible NO synthase sequence over this region. In order to confirm that the human chondrocyte induction was producing a similar iNOS mRNA to that induced in rabbit chondrocytes, RT-PCR was carried out using the primer set BB3 and BB4. PolyA⁺ mRNA (50 ng) from

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induced human cells was used as a template and cDNA was generated by random priming. PCR resulted in a 350 bp band which was purified, cloned and sequenced. Analysis of the sequence demonstrated that the human chondrocyte iNOS cDNA had high (>80%) identity with the murine inducible NOS cDNA over this region.

In order to clone the full-length cDNA for human iNOS, a cDNA library was constructed in lambda ZAPII using oligo dT and random primed polyA⁺ mRNA isolated from induced cells. To maximize the chance of finding a full-length clone a [α^{32} P]-labelled probe was prepared from a 650 bp 5'-fragment of the murine inducible NOS cDNA cloned from mRNA isolated from the cell line J774. This cell line has a cytokine-inducible NOS cDNA sequence that is identical to that described for the RAW 264.7 cell line (our unpublished observations).

Screening the library with the 5'-probe resulted in the identification of several clones, one of which (pBS HSINOS) contained the full-length cDNA for iNOS. DNA sequence analysis of the 4164 bp cDNA clone showed the presence of an open reading frame capable of encoding a protein of 1153 amino acids with a calculated molecular mass of 131,213 daltons. The start site around, the ATG contains a Kozak consensus sequence (TAGAGATGG; Kozak (1991) J. Cell. Biol. 115, 887-903). Comparison of the deduced sequence of the human inducible NO synthase with its murine counterpart shows the proteins to be highly related. The murine enzyme comprises 1144 amino acids with a calculated molecular mass of 130,556 daltons. Overall, the two proteins have 81% identity and 88% similarity as determined by the GAP align program (Trade Name, Wisconsin GCG). Both molecules have consensus recognition sites for the co-factors FAD, FMN and NADPH and in addition have a calmodulin recognition motif, although both enzymes are Ca²⁺ independent.

Subcloning of full-length gene as an XbaI fragment into the expression vector pSVL generated the construct pSVL-NO. Transfection of this construct into CHO cells led to the isolation of a stable cell line expressing human inducible NO

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synthase under the control of a heterologous constitutive promoter (Fig. 2). The expressed NO synthase activity was inhibited by L-NIO (100 μ M) and by L-NMMA (100 μ M).

Example 2

Description of the Drawings

Figure 3 SDS-PAGE of baculovirus/insect cell expressed human inducible NO synthase.

Tracks 1 and 10, Amersham rainbow molecular weight markers; Track 2, total cell lysate (soluble supernatant fraction;) Tracks 3-9 are fractions from an ADP column eluted with 10mM NADPH. The arrow indicates the position of the 135kDa band corresponding to the iNOS. Samples were run on a 10% SDS-PAGE gel.

MATERIALS AND METHODS

Human inducible NO Synthase CDNA

A full-length human iNOS cDNA fragment was cloned as an *Xba*I fragment into the baculovirus transfer vector pVL1393 to generate pVLHINOS. This vector directs the expression of recombinant proteins under the control of the strong polyhedrin promoter. The human iNOS construct pVLMINOS was used to generate recombinant *Autographa californica* baculovirus using the Baculogold transfection kit (Pharmingen).

Maintenance and infection of *Spodoptera frugiperda* insect cells

S. frugiperda (Sf-21) cells were maintained as stirred cultures at 27°C in TC100 medium. Roller cultures of Sf-21 cells (5 x 10⁸ cells/800cm² roller) were infected with human iNOS baculovirus (>10⁸pfu/ml) at a ratio of 5pfu/cell for 24 hours at 27°C.

Preparation of NO Synthase

Cytosol preparations containing iNOS were prepared from 2 x 10⁹ cells as described below. Briefly, the *S. frugiperda* cell pellet was harvested and washed in a buffer

containing 0.1M Hepes pH7.4, 1.0mM dithiothreitol. Cells were resuspended (10^8 /ml) in the same buffer and lysed by 3 freeze-thaw cycles. The resulting lysate was centrifuged at 100,000g for 30 min, and the supernatant mixed at 4°C for 45 mins with one ml of 2'-5' ADP sepharose-4B (Pharmacia), (Charles *et al.*, Biochem. Biophys. Res. Commun., 196, 1481-1489). Following washing, NO synthase activity was eluted from the ADP sepharose with 10mM NADPH (reduced nicotinamide adenine dinucleotide phosphate). Insect cell-derived protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Charles *et al.*, Infect. Immun., 59, 1627-1632).

Enzyme Activity

NO synthase activity was measured spectrophotometrically as described previously (Palmer *et al.*, Biochem. Biophys. Res. Commun., 188, 209-215 and Feelisch *et al.*, Eur. J Pharmac., 139, 19-30). In some experiments the effects of altering the arginine concentrations were investigated as was the inhibition of NO synthase with L-NMMA (NG-monomethyl-L-arginine). The effects of calcium chelation with EGTA (ethylene glycol bis-(β -aminoethylether) N,N,N',N'-tetra acetic acid) and the addition of mammalian calmodulin (bovine, from Sigma) were also determined.

RESULTS

A cDNA clone encoding human inducible NO synthase has been isolated from a λ ZAPII cDNA library and cloned into the baculovirus expression vector pVL1393 as an XbaI fragment. Transfection into *S.frugiperda* (Sf-21) cells results in the expression of NO synthase activity that can be isolated by a freeze-thaw procedure. Fig 3 shows 10% SDS-PAGE gel showing the NADPH elution profile of NO synthase from an ADP sepharose column. Track 2 shows the *S.frugiperda* iNOS cell lysate following high speed centrifugation. No clear band is seen corresponding to recombinant iNOS indicating that the NO synthase is not expressed at high level. This contrasts with

the expression of the neuronal forms of NO synthase that can be expressed at high (15-20% total cell protein) levels in insect cells. Tracks 3-9 show the NADPH elution profile. A major band at 135kDa corresponds to the human iNOS. Lower molecular weight bands seen on the gel cross-react with polyclonal antibody against murine iNOS in western blot experiments (data not shown) suggesting that they represent break-down products of the full-length iNOS.

Kinetic studies on recombinant iNOS shows that it has similar characteristics as its native counterpart, with a similar K_m for L-arginine (Table 1). The V_{max} measurements are made on crude protein and are solely a measure of enzyme expression, demonstrating that more iNOS is being produced in the baculovirus system than the induced mammalian cells. Inhibition studies using the NO synthase inhibitor L-NMMA demonstrated that recombinant and native iNOS have similar IC_{50} values at 30 μ M L-arginine, with a K_i for L-NMMA of 15 μ M. Although chelation of free calcium to a very low concentration by the addition of EGTA (1mM) caused modest inhibition of the recombinant iNOS ($59 \pm 8.3\%$ inhibition, $n=4$) this was reversed by the addition of mammalian calmodulin (500 μ /ml). Thus, in the presence of mammalian calmodulin the recombinant iNOS was inhibited less than 20% by removal of calcium ($18 \pm 8.9\%$ inhibition, $n=4$), consistent with the behaviour of native iNOS (Palmer *et al.*, (1993) *Biochem. Biophys. Res. Commun.*, 193, 398-405; Radomski *et al.* (1991) *Cancer Res.*, 51, 6073-6078; Stuehr *et al.*, (1992) *Adv. Enzymol.*, 65, 287-346).

Table 1: Summary of the characteristics of recombinant human iNOS compared with its native counterpart. The native iNOS was

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measured from IL-1 induced human chondrocytes and megakaryocytes (Meg-01) and a human adenocarcinoma cell-line SW480.

Properties of Expressed Recombinant Human iNOS

	Recombinant Human iNOS	Native Human iNOS
V _{max} (pmol/min per mg)	430 ± 150	220 (IL-1 treated chondrocytes) 180 (SW480 cells) 3 (IL-1 treated Meg-01 cells)
K _m for L-Arg (μM)	4.0 ± 0.38	4 (SW480) 4 (Meg-01)
IC ₅₀ for L-NMMA (μM at 30μM L-Arg)	13 ± 2.0	12 (chondrocytes) 19 (Meg-01)

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4164 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens
 (G) CELL TYPE: chondrocyte

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 226..3687

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

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TACCCCGGGG AGGCAGTGCA GCCAGCTGCA AGCCCCACAG TGAAGAACAT CTGAGCTCAA      180

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Met Ala Cys	
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Pro Trp Lys Phe Leu Phe Lys Thr Lys Phe His Gln Tyr Ala Met Asn	
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GGG GAA AAA GAC ATC AAC AAC AAT GTG GAG AAA GCC CCC TGT GCC ACC	330
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Ser Ser Pro Val Thr Gln Asp Asp Leu Gln Tyr His Asn Leu Ser Lys	
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Gln Gln Asn Glu Ser Pro Gln Pro Leu Val Glu Thr Gly Lys Lys Ser	
55 60 65	
CCA GAA TCT CTG GTC AAG CTG GAT GCA ACC CCA TTG TCC TCC CCA CGG	474
Pro Glu Ser Leu Val Lys Leu Asp Ala Thr Pro Leu Ser Ser Pro Arg	
70 75 80	
CAT GTG AGG ATC AAA AAC TGG GGC AGC GGG ATG ACT TTC CAA GAC ACA	522
His Val Arg Ile Lys Asn Trp Gly Ser Gly Met Thr Phe Gln Asp Thr	
85 90 95	
CTT CAC CAT AAG GCC AAA GGG ATT TTA ACT TGC AGG TCC AAA TCT TGC	570
Leu His His Lys Ala Lys Gly Ile Leu Thr Cys Arg Ser Lys Ser Cys	
100 105 110 115	
CTG GGG TCC ATT ATG ACT CCC AAA AGT TTG ACC AGA GGA CCC AGG GAC	618
Leu Gly Ser Ile Met Thr Pro Lys Ser Leu Thr Arg Gly Pro Arg Asp	
120 125 130	
AAG CCT ACC CCT CCA GAT GAG CTT CTA CCT CAA GCT ATC GAA TTT GTC	666
Lys Pro Thr Pro Pro Asp Glu Leu Leu Pro Gln Ala Ile Glu Phe Val	
135 140 145	
AAC CAA TAT TAC GGC TCC TTC AAA GAG GCA AAA ATA GAG GAA CAT CTG	714
Asn Gln Tyr Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu Glu His Leu	
150 155 160	
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Phe Asp Ala Arg Ser Cys Ser Thr Ala Arg Glu Met Phe Glu His Ile	
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Trp	Asn	Ala	Gln	Leu	Ile	Arg	Tyr	Ala	Gly	Tyr	Gln	Met	Pro	Asp	Gly	
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GAC	CTT	GTG	CTT	GAG	GTG	GCC	ATG	GAA	CAT	CCC	AAA	TAC	GAG	TGG	TTT	1242
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CGG	GAA	CTG	GAG	CTA	AAG	TGG	TAC	GCC	CTG	CCT	GCA	GTG	GCC	AAC	ATG	1290
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Ile	Gln	Ile		Pro	Lys	Leu	Tyr	Thr	Ser	Asn	Val	Thr	Trp	Asp	Pro	
				695				700						705		

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710 715 720	
CTC AGC AGC ATG CAT GCC AAG AAC GTG TTC ACC ATG AGG CTC AAA TCT	2442
Leu Ser Ser Met His Ala Lys Asn Val Phe Thr Met Arg Leu Lys Ser	
725 730 735	
CGG CAG AAT CTA CAA AGT CCG ACA TCC AGC CGT GCC ACC ATC CTG GTG	2490
Arg Gln Asn Leu Gln Ser Pro Thr Ser Ser Arg Ala Thr Ile Leu Val	
740 745 750 755	
GAA CTC TCC TGT GAG GAT GGC CAA GGC CTG AAC TAC CTG CCG GGG GAG	2538
Glu Leu Ser Cys Glu Asp Gly Gln Gly Leu Asn Tyr Leu Pro Gly Glu	
760 765 770	
CAC CTT GGG GTT TGC CCA GGC AAC CAG CCG GCC CTG GTC CAA GGT ATC	2586
His Leu Gly Val Cys Pro Gly Asn Gln Pro Ala Leu Val Gln Gly Ile	
775 780 785	
CTG GAG CGA GTG GTG GAT GGC CCC ACA CCC CAC CAG ACA GTG CGC CTG	2634
Leu Glu Arg Val Val Asp Gly Pro Thr Pro His Gln Thr Val Arg Leu	
790 795 800	
GAG GCC CTG GAT GAG AGT GGC AGC TAC TGG GTC AGT GAC AAG AGG CTG	2682
Glu Ala Leu Asp Glu Ser Gly Ser Tyr Trp Val Ser Asp Lys Arg Leu	
805 810 815	
CCC CCC TGC TCA CTC AGC CAG GCC CTC ACC TAC TTC CTG GAC ATC ACC	2730
Pro Pro Cys Ser Leu Ser Gln Ala Leu Thr Tyr Phe Leu Asp Ile Thr	
820 825 830 835	
ACA CCC CCA ACC CAG CTG CTG CTC CAA AAG CTG GCC CAG GTG GCC ACA	2778
Thr Pro Pro Thr Gln Leu Leu Leu Gln Lys Leu Ala Gln Val Ala Thr	
840 845 850	
GAA GAG CCT GAG AGA CAG AGG CTG GAG GCC CTG TGC CAG CCC TCA GAG	2826
Glu Glu Pro Glu Arg Gln Arg Leu Glu Ala Leu Cys Gln Pro Ser Glu	
855 860 865	
TAC AGC AAG TGG AAG TTC ACC AAC AGC CCC ACA TTC CTG GAG GTG CTA	2874
Tyr Ser Lys Trp Lys Phe Thr Asn Ser Pro Thr Phe Leu Glu Val Leu	
870 875 880	
GAG GAG TTC CCG TCC CTG CGG GTG TCT GCT GGC TTC CTG CTT TCC CAG	2922
Glu Glu Phe Pro Ser Leu Arg Val Ser Ala Gly Phe Leu Leu Ser Gln	
885 890 895	
CTC CCC ATT CTG AAG CCC AGG TTC TAC TCC ATC AGC TCC TCC CGG GAT	2970
Leu Pro Ile Leu Lys Pro Arg Phe Tyr Ser Ile Ser Ser Ser Arg Asp	
900 905 910 915	

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CAC ACG CCC ACA GAG ATC CAC CTG ACT GTG GCC GTG GTC ACC TAC CAC His Thr Pro Thr Glu Ile His Leu Thr Val Ala Val Val Thr Tyr His 920 925 930	3018
ACC CGA GAT GGC CAG GGT CCC CTG CAC CAC GGC GTC TGC AGC ACA TGG Thr Arg Asp Gly Gln Gly Pro Leu His His Gly Val Cys Ser Thr Trp 935 940 945	3066
CTC AAC AGC CTG AAG CCC CAA GAC CCA GTG CCC TGC TTT GTG CGG AAT Leu Asn Ser Leu Lys Pro Gln Asp Pro Val Pro Cys Phe Val Arg Asn 950 955 960	3114
GCC AGC GGC TTC CAC CTC CCC GAG GAT CCC TCC CAT CCT TGC ATC CTC Ala Ser Gly Phe His Leu Pro Glu Asp Pro Ser His Pro Cys Ile Leu 965 970 975	3162
ATC GGG CCT GGC ACA GGC ATC GCG CCC TTC CGC AGT TTC TGG CAG CAA Ile Gly Pro Gly Thr Glu Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln 980 985 990 995	3210
CGG CTC CAT GAC TCC CAG CAC AAG GGA GTG CGG GGA GGC CGC ATG ACC Arg Leu His Asp Ser Gln His Lys Gly Val Arg Gly Gly Arg Met Thr 1000 1005 1010	3258
TTG GTG TTT GGG TGC CGC CGC CCA GAT GAG GAC CAC ATC TAC CAG GAG Leu Val Phe Gly Cys Arg Arg Pro Asp Glu Asp His Ile Tyr Gln Glu 1015 1020 1025	3306
GAG ATG CTG GAG ATG GCC CAG AAG GGG GTG CTG CAT GCG GTG CAC ACA Glu Met Leu Glu Met Ala Gln Lys Gly Val Leu His Ala Val His Thr 1030 1035 1040	3354
GCC TAT TCC CGC CTG CCT GGC AAG CCC AAG GTC TAT GTT CAG GAC ATC Ala Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val Gln Asp Ile 1045 1050 1055	3402
CTG CGG CAG CAG CTG GCC AGC GAG GTG CTC CGT GTG CTC CAC AAG GAG Leu Arg Gln Gln Leu Ala Ser Glu Val Leu Arg Val Leu His Lys Glu 1060 1065 1070 1075	3450
CCA GGC CAC CTC TAT GTT TGC GGG GAT GTG CGC ATG GCC CGG GAC GTG Pro Gly His Leu Tyr Val Cys Gly Asp Val Arg Met Ala Arg Asp Val 1080 1085 1090	3498
GCC CAC ACC CTG AAG CAG CTG GTG GCT GCC AAG CTG AAA TTG AAT GAG Ala His Thr Leu Lys Gln Leu Val Ala Ala Lys Leu Lys Leu Asn Glu 1095 1100 1105	3546
GAG CAG GTC GAG GAC TAT TTC TTT CAG CTC AAG AGC CAG AAG CGC TAT Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln Lys Arg Tyr 1110 1115 1120	3594
CAC GAA GAT ATC TTT GGT GCT GTA TTT CCT TAC GAG GCG AAG AAG GAC His Glu Asp Ile Phe Gly Ala Val Phe Pro Tyr Glu Ala Lys Lys Asp 1125 1130 1135	3642
AGG GTG GCG GTG CAG CCC AGC AGC CTG GAG ATG TCA GCG CTC TGAGGGCCTA Arg Val Ala Val Gln Pro Ser Ser Leu Glu Met Ser Ala Leu 1140 1145 1150	3694

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CAGGAGGGGT TAAAGCTGCC GGCACAGAAC TTAAGGATGG AGCCAGCTCT GCATTATCTG 3754
 AGGTCACAGG GCCTGGGGAG ATGGAGGAAA GTGATATCCC CCAGCCTCAA GTCTTATTTT 3814
 CTCAACGTTG CTCCCCATCA AGCCCTTTAC TTGACCTCCT AACAAAGTAGC ACCCTGGATT 3874
 GATCGGAGCC TCCTCTCTCA AACTGGGGCC TCCTGGGTCC CTTGGAGACA AAATCTTAAA 3934
 TGCCAGGCCT GGCAAGTGGG TGAAAGATGG AACTTGCTGC TGAGTGCACC ACTTCAAGTG 3994
 ACCACCAGGA GGTGCTATCG CACCACTGTG TATTAACTG CCTTGTGTAC AGTTATTTAT 4054
 GCCTCTGTAT TTAAAAACT AACACCCAGT CTGTTCCCCA TGGCCACTTG GGTCTTCCCT 4114
 GTATGATTCC TTGATGGAGA TATTACATG AATTGCATTT TACTTTAATC 4164

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1153 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Cys Pro Trp Lys Phe Leu Phe Lys Thr Lys Phe His Gln Tyr
 1 5 10 15
 Ala Met Asn Gly Glu Lys Asp Ile Asn Asn Asn Val Glu Lys Ala Pro
 20 25 30
 Cys Ala Thr Ser Ser Pro Val Thr Gln Asp Asp Leu Gln Tyr His Asn
 35 40 45
 Leu Ser Lys Gln Gln Asn Glu Ser Pro Gln Pro Leu Val Glu Thr Gly
 50 55 60
 Lys Lys Ser Pro Glu Ser Leu Val Lys Leu Asp Ala Thr Pro Leu Ser
 65 70 75 80
 Ser Pro Arg His Val Arg Ile Lys Asn Trp Gly Ser Gly Met Thr Phe
 85 90 95
 Gln Asp Thr Leu His His Lys Ala Lys Gly Ile Leu Thr Cys Arg Ser
 100 105 110
 Lys Ser Cys Leu Gly Ser Ile Met Thr Pro Lys Ser Leu Thr Arg Gly
 115 120 125
 Pro Arg Asp Lys Pro Thr Pro Pro Asp Glu Leu Leu Pro Gln Ala Ile
 130 135 140
 Glu Phe Val Asn Gln Tyr Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu
 145 150 155 160
 Glu His Leu Ala Arg Val Glu Ala Val Thr Lys Glu Ile Glu Thr Thr
 165 170 175

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Gly Thr Tyr Gln Leu Thr Gly Asp Glu Leu Ile Phe Ala Thr Lys Gln
 180 185 190
 Ala Trp Arg Asn Ala Pro Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn
 195 200 205
 Leu Gln Val Phe Asp Ala Arg Ser Cys Ser Thr Ala Arg Glu Met Phe
 210 215 220
 Glu His Ile Cys Arg His Val Arg Tyr Ser Thr Asn Asn Gly Asn Ile
 225 230 235 240
 Arg Ser Ala Ile Thr Val Phe Pro Gln Arg Ser Asp Gly Lys His Asp
 245 250 255
 Phe Arg Val Trp Asn Ala Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met
 260 265 270
 Pro Asp Gly Ser Ile Arg Gly Asp Pro Ala Asn Val Glu Phe Thr Gln
 275 280 285
 Leu Cys Ile Asp Leu Gly Trp Lys Pro Lys Tyr Gly Arg Phe Asp Val
 290 295 300
 Val Pro Leu Val Leu Gln Ala Asn Gly Arg Asp Pro Glu Leu Phe Glu
 305 310 315 320
 Ile Pro Pro Asp Leu Val Leu Glu Val Ala Met Glu His Pro Lys Tyr
 325 330 335
 Glu Trp Phe Arg Glu Leu Glu Leu Lys Trp Tyr Ala Leu Pro Ala Val
 340 345 350
 Ala Asn Met Leu Leu Glu Val Gly Gly Leu Glu Phe Pro Gly Cys Pro
 355 360 365
 Phe Asn Gly Trp Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Phe Cys
 370 375 380
 Asp Val Gln Arg Tyr Asn Ile Leu Glu Glu Val Gly Arg Arg Met Gly
 385 390 395 400
 Leu Glu Thr His Lys Leu Ala Ser Leu Trp Lys Asp Gln Ala Val Val
 405 410 415
 Glu Ile Asn Ile Ala Val Leu His Ser Phe Gln Lys Gln Asn Val Thr
 420 425 430
 Ile Met Asp His His Ser Ala Ala Glu Ser Phe Met Lys Tyr Met Gln
 435 440 445
 Asn Glu Tyr Arg Ser Arg Gly Gly Cys Pro Ala Asp Trp Ile Trp Leu
 450 455 460
 Val Pro Pro Met Ser Gly Ser Ile Thr Pro Val Phe His Gln Glu Met
 465 470 475 480
 Leu Asn Tyr Val Leu Ser Pro Phe Tyr Tyr Tyr Gln Val Glu Ala Trp
 485 490 495
 Lys Thr His Val Trp Gln Asp Glu Lys Arg Arg Pro Lys Arg Arg Glu
 500 505 510

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Ile Pro Leu Lys Val Leu Val Lys Ala Val Leu Phe Ala Cys Met Leu
 515 520 525
 Met Arg Lys Thr Met Ala Ser Arg Val Arg Val Thr Ile Leu Phe Ala
 530 535 540
 Thr Glu Thr Gly Lys Ser Glu Ala Leu Ala Trp Asp Leu Gly Ala Leu
 545 550 555 560
 Phe Ser Cys Ala Phe Asn Pro Lys Val Val Cys Met Asp Lys Tyr Arg
 565 570 575
 Leu Ser Cys Leu Glu Glu Glu Arg Leu Leu Leu Val Val Thr Ser Thr
 580 585 590
 Phe Gly Asn Gly Asp Cys Pro Gly Asn Gly Glu Lys Leu Lys Lys Ser
 595 600 605
 Leu Phe Met Leu Lys Glu Leu Asn Asn Lys Phe Arg Tyr Ala Val Phe
 610 615 620
 Gly Leu Gly Ser Ser Met Tyr Pro Arg Phe Cys Ala Phe Ala His Asp
 625 630 635 640
 Ile Asp Gln Lys Leu Ser His Leu Gly Ala Ser Gln Leu Thr Pro Met
 645 650 655
 Gly Glu Gly Asp Glu Leu Ser Gly Gln Glu Asp Ala Phe Arg Ser Trp
 660 665 670
 Ala Val Gln Thr Phe Lys Ala Ala Cys Glu Thr Phe Asp Val Arg Gly
 675 680 685
 Lys Gln His Ile Gln Ile Pro Lys Leu Tyr Thr Ser Asn Val Thr Trp
 690 695 700
 Asp Pro His His Tyr Arg Leu Val Gln Asp Ser Gln Pro Leu Asp Leu
 705 710 715 720
 Ser Lys Ala Leu Ser Ser Met His Ala Lys Asn Val Phe Thr Met Arg
 725 730 735
 Leu Lys Ser Arg Gln Asn Leu Gln Ser Pro Thr Ser Ser Arg Ala Thr
 740 745 750
 Ile Leu Val Glu Leu Ser Cys Glu Asp Gly Gln Gly Leu Asn Tyr Leu
 755 760 765
 Pro Gly Glu His Leu Gly Val Cys Pro Gly Asn Gln Pro Ala Leu Val
 770 775 780
 Gln Gly Ile Leu Glu Arg Val Val Asp Gly Pro Thr Pro His Gln Thr
 785 790 795 800
 Val Arg Leu Glu Ala Leu Asp Glu Ser Gly Ser Tyr Trp Val Ser Asp
 805 810 815
 Lys Arg Leu Pro Pro Cys Ser Leu Ser Gln Ala Leu Thr Tyr Phe Leu
 820 825 830

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Asp Ile Thr Thr Pro Pro Thr Gln Leu Leu Leu Gln Lys Leu Ala Gln
 835 840 845
 Val Ala Thr Glu Glu Pro Glu Arg Gln Arg Leu Glu Ala Leu Cys Gln
 850 855 860
 Pro Ser Glu Tyr Ser Lys Trp Lys Phe Thr Asn Ser Pro Thr Phe Leu
 865 870 875 880
 Glu Val Leu Glu Glu Phe Pro Ser Leu Arg Val Ser Ala Gly Phe Leu
 885 890 895
 Leu Ser Gln Leu Pro Ile Leu Lys Pro Arg Phe Tyr Ser Ile Ser Ser
 900 905 910
 Ser Arg Asp His Thr Pro Thr Glu Ile His Leu Thr Val Ala Val Val
 915 920 925
 Thr Tyr His Thr Arg Asp Gly Gln Gly Pro Leu His His Gly Val Cys
 930 935 940
 Ser Thr Trp Leu Asn Ser Leu Lys Pro Gln Asp Pro Val Pro Cys Phe
 945 950 955 960
 Val Arg Asn Ala Ser Gly Phe His Leu Pro Glu Asp Pro Ser His Pro
 965 970 975
 Cys Ile Leu Ile Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe
 980 985 990
 Trp Gln Gln Arg Leu His Asp Ser Gln His Lys Gly Val Arg Gly Gly
 995 1000 1005
 Arg Met Thr Leu Val Phe Gly Cys Arg Arg Pro Asp Glu Asp His Ile
 1010 1015 1020
 Tyr Gln Glu Glu Met Leu Glu Met Ala Gln Lys Gly Val Leu His Ala
 1025 1030 1035 1040
 Val His Thr Ala Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val
 1045 1050 1055
 Gln Asp Ile Leu Arg Gln Gln Leu Ala Ser Glu Val Leu Arg Val Leu
 1060 1065 1070
 His Lys Glu Pro Gly His Leu Tyr Val Cys Gly Asp Val Arg Met Ala
 1075 1080 1085
 Arg Asp Val Ala His Thr Leu Lys Gln Leu Val Ala Ala Lys Leu Lys
 1090 1095 1100
 Leu Asn Glu Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln
 1105 1110 1115 1120
 Lys Arg Tyr His Glu Asp Ile Phe Gly Ala Val Phe Pro Tyr Glu Ala
 1125 1130 1135
 Lys Lys Asp Arg Val Ala Val Gln Pro Ser Ser Leu Glu Met Ser Ala
 1140 1145 1150
 Leu

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGATCCGG NACNGGNATH GCNCCNTT

28

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGAATTCNC CRCANACRTA DATRTG

26

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACGGAGAAGC TTAGATCTGG AGCAGAAGTG

30

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGCAGGTTG GACCACTGGA TCCTGCCGAT

30

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CLAIMS

1. A nitric oxide (NO) synthase having a sequence at least 85% identical to the sequence of SEQ ID NO: 2.
2. A NO synthase according to Claim 1 having a sequence at least 90% identical to the sequence of SEQ ID NO:2.
3. A NO synthase according to Claim 2 having a sequence at least 95% identical to the sequence of SEQ ID NO:2.
4. A NO synthase according to Claim 3 having a sequence at least 99% identical to the sequence of SEQ ID NO:2.
5. An NO synthase according to claim any one of claims 1 to 4 which is of human origin.
6. An NO synthase according to any preceeding claim in substantially pure form.
7. A DNA molecule encoding an NO synthase according to any preceeding claim.
8. A replicable expression vector containing a DNA molecule according to Claim 7.
9. A host cell transformed or transfected with a vector as claimed in claim 8.
10. A method of producing an NO synthase according to any one of claims 1 to 6, which comprises
 - (a) culturing a host cell according to claim 9 under conditions in which the cell expresses the NO synthase; and
 - (b) recovering the NO synthase from the culture.
11. An antisense oligonucleotide having a sequence at least 85% identical to the sequence of ID NO:1.
12. An antisense oligonucleotide having a sequence at least 95% identical to the sequence of ID NO:1.
13. An antisense oligonucleotide according to either of claims 11 and 12 in which the fragment is 12 to 30 nucleotides in length.
14. An antibody specific for an NO synthase according to any one of claims 1 to 6.
15. A method of detecting or quantitatively determining in a sample an NO synthase according to any one of claims 1 to 6, which comprises

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(a) contacting the sample with an antibody as claimed in claim 14; and

(b) detecting or quantitatively determining the binding of the antibody.

16. A method for identifying a substrate which inhibits or stimulates an NO synthase according to any one of claims 1 to 6, which comprises

- (a) incubating the NO synthase with the substrate;
- (b) measuring the activity of the NO synthase; and
- (c) comparing the activity measured in (b) above with the activity of the NO synthase in the absence of the substrate.

17. A substrate identified by method according to claim 16.

18. An enzyme-substrate complex which comprises an NO synthase according to any of claims 1 to 6 and a substrate according to Claim 17.

19. A pharmaceutical formulation which comprises one or more of a substrate according to Claim 17, a NO synthase according to any one of Claims 1 to 6 or an antibody according to Claim 14, in combination with a pharmaceutically acceptable carrier or diluent therefor, and optionally one or more further therapeutic agents.

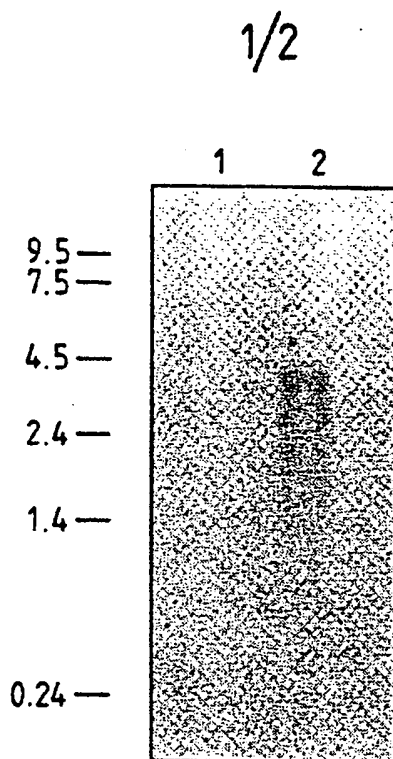


Fig. 1

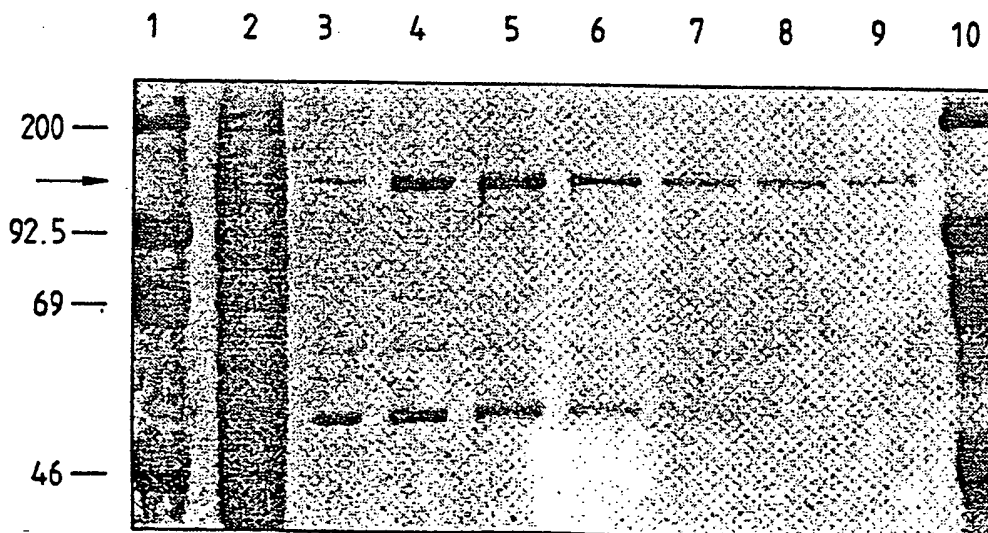


Fig. 3

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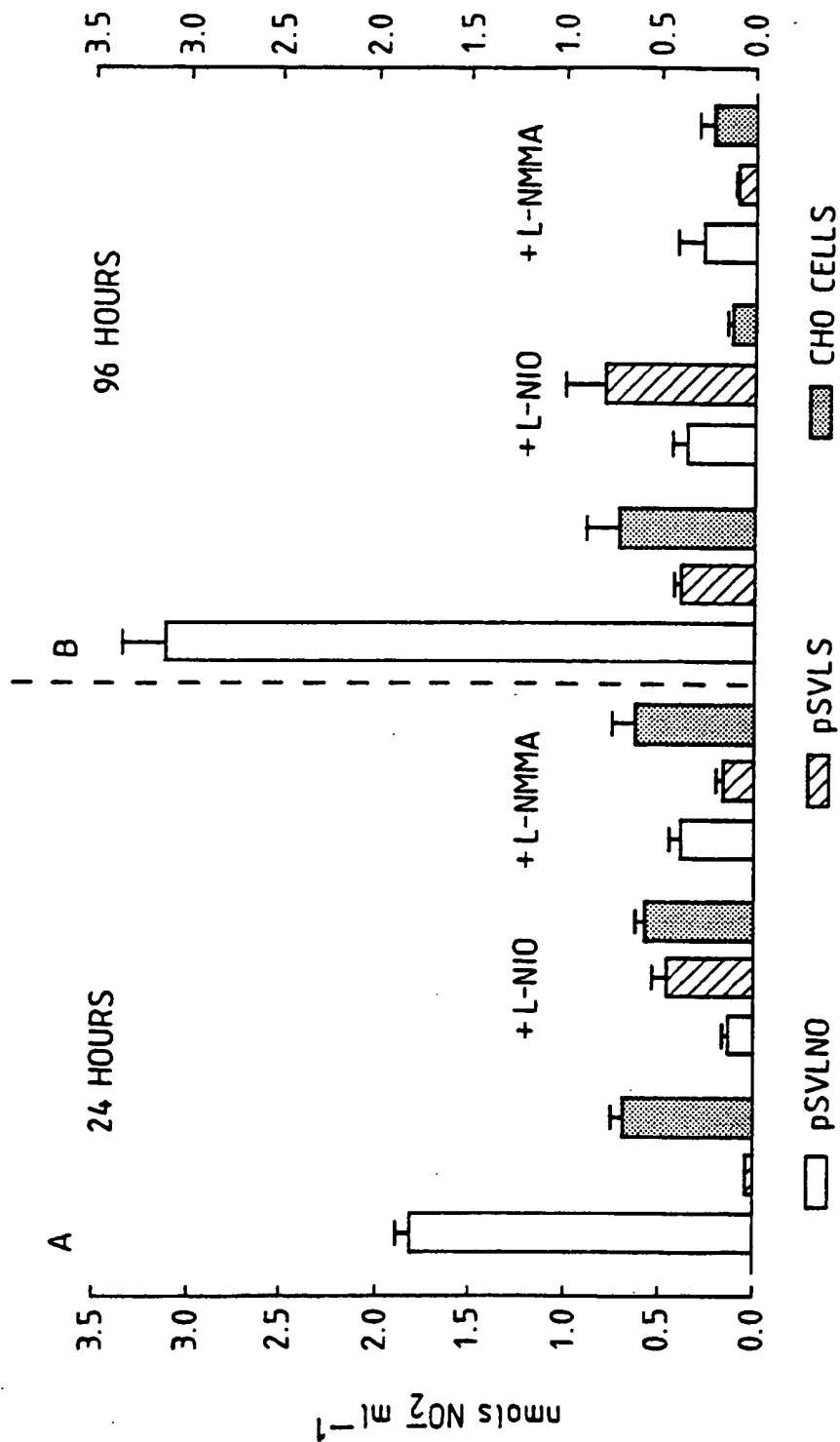


Fig. 2

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INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/GB 94/00621

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/53 C12N15/85 C12N9/02 C07K15/06 A61K39/395
 A61K37/50 C12Q1/527 C12Q1/68 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. BIOL. CHEM. vol. 267, no. 9, 25 March 1992 pages 6370 - 6374 LYONS ET AL. 'Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line' cited in the application ---	1,6-11, 13
X	BIOCHEM. BIOPHYS. RES. COMMUN. vol. 191, no. 1, 26 February 1993 pages 89 - 94 NUNOKAWA ET AL. 'Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells' --- -/--	1,6,7, 11,13,19

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

* "A" document defining the general state of the art which is not considered to be of particular relevance

* "E" earlier document but published on or after the international filing date

* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

* "O" document referring to an oral disclosure, use, exhibition or other means

* "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* "&" document member of the same patent family

Date of the actual completion of the international search

29 July 1994

Date of mailing of the international search report

- 4. 08. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Gac, G

INTERNATIONAL SEARCH REPORT

Inter: International Application No

PCT/GB 94/00621

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROC. NATL ACAD. SCI. vol. 89 , 1 August 1992 pages 6711 - 6715 LOWENSTEIN ET AL. 'Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme' cited in the application ---	1,6-11
P,X	PROC. NATL ACAD. SCI. vol. 90 , December 1993 pages 11419 - 11423 CHARLES ET AL. 'Cloning, characterization and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte' ---	1-13
P,X	ARTHRIT. RHEUMAT. vol. 36, no. SUP9 , September 1993 page S189 REDICKE ET AL. 'Human articular chondrocytes induced by proinflammatory cytokines are the major intraarticular source of nitric oxide (NO) and NO mediates the IL-1 induction of cGMP' -----	1-6, 16-19

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